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Identification of CD72 as a Lymphocyte Receptor for the Class IV Semaphorin CD100: A Novel Mechanism for Regulating B Cell Signaling

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Summary

We have identified the lymphocyte semaphorin CD100/Sema4D as a CD40-inducible molecule by subtractive cDNA cloning. CD100 stimulation significantly enhanced the effects of CD40 on B cell responses. Administration of soluble CD100 markedly accelerated in vivo antigen-specific antibody responses. CD100 receptors with different binding affinities were detected on renal tubular cells ($K_d = \sim 1 \times 10^{-9}$ M) and lymphocytes ($K_d = \sim 3 \times 10^{-7}$ M). Expression cloning revealed that the CD100 receptor on lymphocytes is CD72, a negative regulator of B cell responsiveness. CD72 thus represents a novel class of semaphorin receptors. CD100 stimulation induced tyrosine dephosphorylation of CD72 and dissociation of SHP-1 from CD72. Our findings indicate that CD100 plays a critical role in immune responses by the novel mechanism of turning off negative signaling by CD72.

Introduction

The semaphorin family consists of a large number of phylogenetically conserved proteins, carrying a long (approximately 500 amino acid residues) stretch of conserved "Sema domain" in the amino terminus (Yu and Kolodkin, 1999). Based on additional structural features, the family has been divided into eight classes (Semaphorin Nomenclature Committee, 1999). Many members of the semaphorin family are critically involved in axon steering, fasciculation, branching, and synapse formation by functioning as chemorepellents during neuronal development (Kolodkin et al., 1992, 1993; Luo et al., 1993; Yu and Kolodkin, 1999). Although the functions of semaphorins have been initially addressed with respect to neuronal guidance, it is becoming clear that semaphorins play important roles in organogenesis, vascularization, and angiogenesis and the progression of cancers (Kitsukawa et al., 1995; Behar et al., 1996; Sekido et al., 1996). Recently, neuropilins and plexins have been shown to be receptors for some members of this family and to mediate the functions of semaphorins in the nervous system (He and Tessier Lavigne, 1997; Kolodkin et al., 1997; Comeau et al., 1998; Winberg et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999).

The 150 kDa transmembrane protein CD100/Sema4D belongs to group IV of the semaphorin family. CD100 has been shown to be expressed in a broad range of tissues from embryos to adults, including neural tissues, kidney, lung, and heart (Furuyama et al., 1996; Hall et al., 1996). In addition, CD100 is the only semaphorin member known to be expressed physiologically in the immune system. Antibody crosslinking of CD100 has been shown to provide a proliferative signal to T cells in the presence of submitogenic doses of anti-CD3 or anti-CD2 antibodies (Herold et al., 1995), suggesting that CD100 may be involved in T cell activation. Association of tyrosine phosphatases with CD100 has also been suggested (Herold et al., 1996; Billard et al., 2000). On the other hand, CD100-expressing transfectants have been reported to promote aggregation and survival of B cells in vitro, suggesting that CD100, like other semaphorin proteins, can function as a ligand through a functional receptor on lymphocytes (Hall et al., 1996). Recently, plexin-B1, which is widely expressed with prominent levels in the fetal brain and kidney, has been reported to be a high-affinity receptor for CD100 (Tamagnone et al., 1999). However, it is unknown whether plexin-B1 is also a functional receptor for CD100 in the immune system.

CD72 is a 45 kDa type II transmembrane protein that belongs to the C-type lectin family (Nakayama et al., 1989; Von Hoegen et al., 1990). CD72 is expressed throughout B cell differentiation from the earliest B cell progenitors to mature B cells but is downregulated upon terminal differentiation into plasma cells (Gordon, 1994; Tutt Landolfi and Parnes, 1997). In some mouse strains, CD72 is also expressed on subpopulations of T cells (Robinson et al., 1997). Engagement of CD72 can transform a subset of small resting B cells into blast cells and induce proliferation of activated B cells (Subbarao and Mosier, 1984; Yakura et al., 1986). Anti-CD72 monoclonal antibodies (mAbs) have been shown to block BCR-mediated cell death, promote B cell survival and proliferation, increase MHC class II expression, and enhance production and release of CD23 in B cells (Gordon et al., 1991; Katira et al., 1992; Gordon, 1994; Nomura et al., 1996; Tutt Landolfi and Parnes, 1997). Many of the effects of anti-CD72 mAbs on resting B cells are significantly enhanced by CD40 stimulation (Gordon et al., 1991; Gordon, 1994; Tutt Landolfi and Parnes, 1997).

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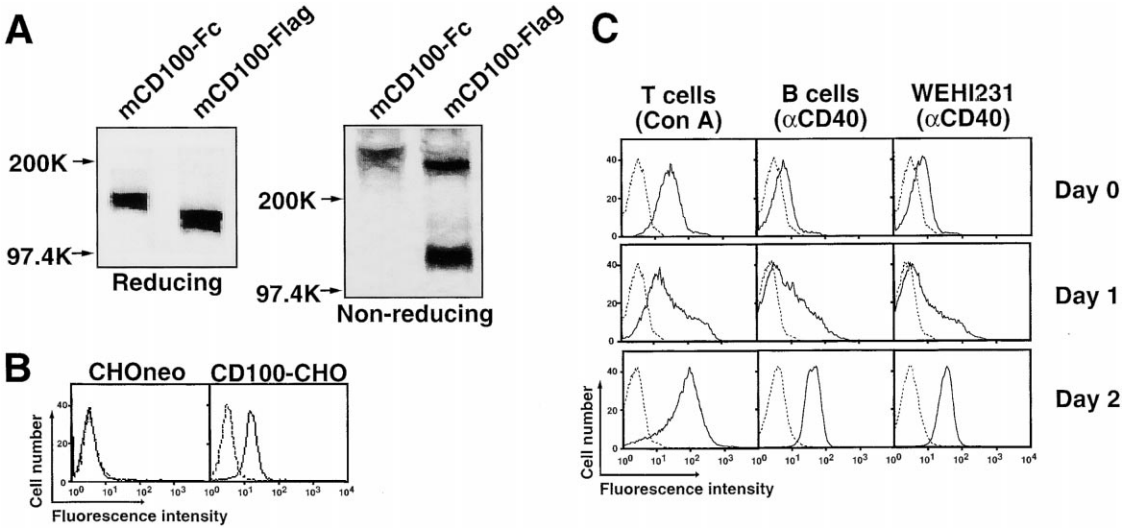

Figure 1. Expression of CD100 Is Upregulated on Activated T and B Cells

(A) Analysis of purified mCD100-Fc and mCD100-Flag by SDS-PAGE. Two micrograms of purified mCD100-Fc or mCD100-Flag was separated by gradient PAGE (4%–20%) in the presence of 0.1% SDS under reducing or nonreducing conditions and visualized by silver staining. Molecular weight markers (kDa) (Rainbow markers, Amersham) are shown on the left.

(B) Anti-mCD100 mAb binds specifically to CD100-CHO cells. Stable transfectants of CHOneo and CD100-CHO were established as described in Experimental Procedures. CHOneo or CD100-CHO were stained with biotinylated anti-mCD100 mAb (BMA-12, rat IgG1) (solid line) or biotinylated isotype-matched controls (dotted line) plus FITC-conjugated streptavidin.

(C) Splenic T cells, small resting B cells, or WEHI-231 cells were stained with biotinylated anti-mCD100 mAb (BMA-12) (solid line) or biotinylated isotype-matched controls (dotted line) plus FITC-conjugated streptavidin before and 1 or 2 days after stimulation with Con A (2 μg/ml) or anti-CD40 mAb (HM40-3, 1 μg/ml) and then analyzed by flow cytometry.

In some situations, IL-4 and CD72 signals act in synergy with one another (Yakura et al., 1986). In addition, it has been shown that anti-CD72 mAbs induce tyrosine phosphorylation of phospholipase C-γ and CD19 and activate Lyn, Blk, and Btk kinases (Venkataraman et al., 1998a, 1998b). These observations imply that CD72 transmits positive signals for B cell activation. On the other hand, cumulative evidence also suggests a potential role of CD72 as a negative regulator of B cell responses. The cytoplasmic domain of CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Adachi et al. (1998) have shown that crosslinking of the BCR enhances tyrosine phosphorylation of CD72 and association of CD72 with the protein tyrosine phosphatase SHP-1, suggesting that CD72 might negatively regulate B cell activation. Consistent with this finding, a recent study from one of our labs has shown that B cells from CD72-deficient mice are hyperproliferative in response to various stimuli and have a more rapid Ca^{2+} response following BCR stimulation (Pan et al., 1999). Furthermore, preligation of CD72 with anti-CD72 mAb blocks tyrosine phosphorylation of CD72 and association of SHP-1 with CD72, resulting in inhibition of BCR-induced growth arrest/apoptosis signals (Wu et al., 1998). These findings suggest that binding of a putative natural ligand to CD72 may switch its signaling from positive to negative or vice versa, thereby controlling the magnitude of B cell responses.

Here, we identify CD72 as a functional lymphocyte receptor for CD100 and demonstrate that CD100 stimulation turns off negative signaling effects of CD72 by inducing tyrosine dephosphorylation of CD72 and dissociation of SHP-1 from CD72.

Results

CD100 Expression Is Upregulated on B Cells by Physiological Stimuli

In a screen for the genes induced in CD40-stimulated B cells, we isolated the cDNA encoding the mouse homolog of human CD100 (Furuyama et al., 1996; Hall et al., 1996) (see Experimental Procedures). To investigate the function and expression of CD100, we first prepared soluble CD100 protein consisting of the full length of the putative extracellular region of mouse CD100 fused with either human IgG1 Fc (mCD100-Fc) or with Flag-peptide (mCD100-Flag). As shown in Figure 1A, a band of approximately 150 kDa was observed for mCD100-Fc under reducing conditions, and dimer formation was apparent under nonreducing conditions; however, homodimerization of mCD100-Flag was rather unstable, although CD100 containing its transmembrane and cytoplasmic domains has been reported to form homodimers (Furuyama et al., 1996; Hall et al., 1996). To analyze the cell surface expression of mCD100 on various types of cells, we generated several anti-mouse CD100 monoclonal antibodies (anti-mCD100 mAb) by immunizing rats with mCD100-Fc and screening hybridomas with mCD100-Flag. As shown in Figure 1B, anti-mCD100 mAb (BMA-12; rat IgG1) specifically bound to mCD100-expressing CHO cell transfectants (CD100-CHO) but not to CHO cells transfected with the neomycin resistance plasmid alone (CHOneo). Flow cytometric analysis using anti-mCD100 mAb confirmed that mCD100 was expressed abundantly on T cells and weakly on B cells (Figure 1C), as reported for human CD100 (Delaire et al., 1998). CD100 expression was considerably upregulated

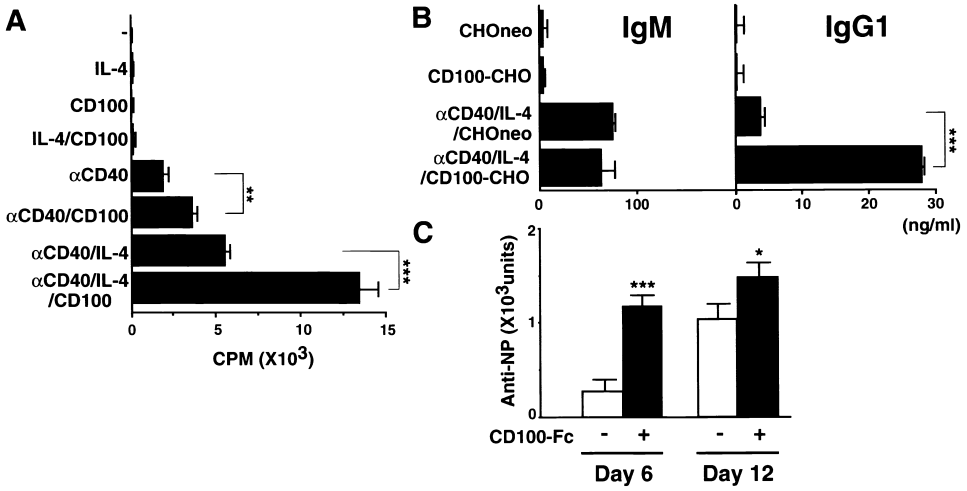

Figure 2. The Effects of CD100 on B Cell Responses

(A) The proliferative effects of CD100 on small resting splenic B cells. Small resting B cells purified from C57BL/6 mice were stimulated with or without anti-CD40 mAb (1 μg/ml), IL-4 (10 U/ml), and mCD100-Fc (20 μg/ml), as indicated, for 3 days. Cells were pulsed with 2 μCi of [^{3}H]thymidine for the last 16 hr of the culture period and then [^{3}H]thymidine uptake was measured.

(B) In vitro IgG1 production induced by CD40 is enhanced by CD100 stimulation. Small resting B cells were stimulated with or without anti-CD40 mAb (1 μg/ml) and IL-4 (10 U/ml) in the presence of paraformaldehyde fixed CHOneo or CD100-CHO cells for 7 days. Concentrations of IgM and IgG1 in the culture supernatants were determined by ELISA.

(C) The effects of mCD100-Fc treatment on in vivo antigen-specific antibody responses. Purified human IgG1 myeloma protein (white bars) or mCD100-Fc (black bars) was injected at 100 μg/day for 12 days after intraperitoneal immunization with 100 μg alum-precipitated NP-CGG. Serum titers of anti-NP-specific IgG1 antibodies were assayed by ELISA using NP_{12}-BSA. The concentrations of anti-NP IgG1 antibodies were estimated by comparison to standard curves created from pooled sera of C57BL/6 mice 12 days after immunization. The concentrations of anti-NP IgG1 present in a 1:1000 dilution of pooled immune sera of C57BL/6 mice was defined as one arbitrary unit (Uchida et al., 1999). The values shown are the means ± SD of ten mice per group. *$p < 0.05$, **$p < 0.01$, **$p < 0.005$. Each value was statistically analyzed by unpaired t test.

by Con A stimulation of T cells and CD40 stimulation of splenic B cells and WEHI-231 cells (Figure 1C). As expected from our cloning methodology of *CD100* cDNA, the upregulation of surface CD100 on B cells correlated with increased *CD100* mRNA (data not shown).

CD100 Stimulation Synergistically Enhances CD40-Mediated B Cell Responses

We expected that CD100 might function as a ligand for a cell surface receptor, like other members of the semaphorin family. We therefore examined effects of CD100 on in vitro B cell responses in which CD40 signaling has been shown to play a role. Stimulation of normal splenic B cells with mCD100-Fc enhanced B cell proliferative responses to anti-CD40 mAb plus IL-4 (Figure 2A). Moreover, addition of CD100-expressing CHO cells significantly increased IgG1 but not IgM production of B cells stimulated with anti-CD40 mAb and IL-4 (Figure 2B). These results indicate that CD100 exerts synergistic effects on CD40-induced B cell responses.

To determine whether CD100 plays a role in antigen-specific antibody production in vivo, mice were immunized with a T-dependent antigen, 4-hydroxy-3-nitrophenylacetyl-chicken-γ-globulin conjugates (NP-CGG), and then treated with soluble mCD100-Fc. As shown in Figure 2C, mice treated with soluble mCD100-Fc exhibited significantly enhanced NP-specific IgG1 antibody levels even 6 days after immunization, which was almost comparable to those seen in untreated mice 12 days later. Collectively, the effects of CD100 stimulation on in vitro and in vivo antibody responses indicate that the lymphocyte semaphorin CD100 plays a critical role in humoral immune responses.

Two Types of Receptors for CD100 with Different Binding Kinetics

Human plexin-B1 has been shown to be a high-affinity receptor for human CD100 (Tamagnone et al., 1999). The expression of plexin-B1 is reported to be detected in a broad range of fetal and adult tissues, with prominent levels in the fetal kidney and brain (Maestrini et al., 1996). The expression of CD100 has also been observed in a broad range of tissues, including brain, kidney, and lymphocytes (Furuyama et al., 1996; Hall et al., 1996). We examined the distribution of CD100 binding sites using various mouse tissue-derived cell lines. Through binding analysis using soluble mCD100-Fc, we found at least two types of receptors for CD100. As shown in Figure 3A, mCD100-Fc bound to the Prox24 cell line (derived from mouse renal tubular cells) with high-affinity binding kinetics ($K_d = \sim 1 \times 10^{-9}$ M, corresponding to the affinity reported for the human CD100–human plexin-B1 interaction). In contrast, mCD100-Fc bound to WEHI-231 cells with lower affinity binding kinetics ($K_d = \sim 3 \times 10^{-7}$ M). Mouse plexin-B1 has not been described previously. We isolated a mouse *plexin-B1* cDNA fragment from a Prox24 cDNA library by PCR cloning using degenerate oligonucleotide primers based on conserved sequences among members of the plexin family (data not

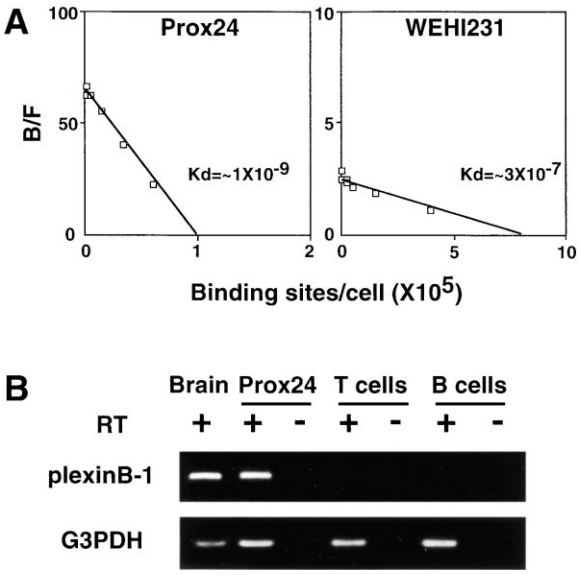

Figure 3. Two Types of Receptors for CD100 with Different Binding Kinetics

(A) Scatchard analysis of ^{125}I mCD100-Fc binding to Prox24 cells or WEHI-231 cells. Prox24 cells or WEHI-231 cells were incubated in the presence of Fc block as triplicate samples in the presence or absence of excess mCD100-Fc for 2 hr. The cell pellet and supernatant were assessed for bound and free ^{125}I cpm, respectively. CD40-Fc protein did not show specific binding under those experimental conditions (data not shown). Values for K_d were obtained by Scatchard analysis as described (Hara and Miyajima, 1992).

(B) RT–PCR for expression of murine *plexin-B1*. Several cDNA fragments of murine *plexin-B1* were isolated by degenerate PCR cloning as described in Experimental Procedures. RNA was isolated from mouse brain, Prox24 cells, and splenic T cells and B cells and then treated with DNase I to exclude genomic DNA. For negative controls, RT^- samples were applied to PCR amplifications.

shown). We examined the expression of *plexin-B1* in mouse lymphocytes by RT–PCR. Although *plexin-B1* transcripts were highly expressed in Prox24 cells and brain, the expression of *plexin-B1* could not be detected in primary mature T and B cells (Figure 3B). The effects of CD100 on B cell responses that we observed here imply that lymphocytes express functional receptors for CD100 that are distinct from plexin-B1. We thus stained various cells with biotinylated mCD100-Fc to search for CD100 receptors on lymphocytes. Splenic B cells as well as WEHI-231 B cells were specifically stained by mCD100-Fc (Figures 4A and 4B). In addition, binding of biotinylated mCD100-Fc was detected on activated splenic T cells and T cell hybridomas but not on resting splenic T cells or thymocytes (Figures 4C–4F).

Expression Cloning Reveals that CD72 Is a Receptor for CD100

For expression cloning of the CD100 receptor on lymphocytes, we constructed a cDNA library from Con A–stimulated T cell hybridoma 2B4 cells, since these abundantly express CD100 binding sites (Figure 4F). Plasmid DNA from the entire library was introduced into COS7 cells. The transfected COS7 cells were allowed to bind biotinylated mCD100-Fc or biotinylated human immunoglobulin Fc fractions followed by magnetic beads conjugated with streptavidin. Cells binding mCD100-Fc were enriched by magnetic sorting. A discrete band corresponding to a 1.4 kb insert appeared after the fourth round of sorting, whereas no bands were apparent with cells binding human immunoglobulin Fc fractions (Figure 5A). We sequenced the 1.4 kb cDNA inserts of these clones and found that they were full-length cDNAs encoding mouse CD72. CD72 was originally defined as an allotypic determinant on mouse B lymphocytes (Sato and Boyse, 1976). The cDNA of *CD72* we isolated here was identical to the *b* allele (Robinson et al., 1992), which is known to be expressed on a subset of T cells and to be further inducible by T cell activation (Robinson et al., 1997). This is in good agreement with our observation that mCD100-Fc binds not only to B cells but also to activated T cells. As shown in Figure 5B, biotinylated mCD100-Fc specifically bound to CD72-expressing CHO cell transfectants (CD72-CHO) but not to control transfectants (CHOneo). Anti-CD72 mAb specifically blocked the binding of mCD100-Fc to B cells (Figure 5C). Furthermore, mCD100-Fc did not bind to CD72-deficient B cells (Figure 5D), nor did it enhance their proliferation or immunoglobulin production induced by anti-CD40 plus IL-4 (Figure 5F). mCD100-Fc also failed to bind Con A–activated T cells from CD72-deficient mice (data not shown). The results demonstrate that CD100 binds specifically to and exerts its function through CD72.

Properties of CD100–CD72 Binding

To investigate the properties of binding of CD100 to CD72, we prepared soluble recombinant CD72 protein consisting of the full length of the extracellular region of CD72 and Flag-peptide (CD72-Flag). As shown in Figure 6A, SDS-PAGE of purified recombinant CD72-Flag protein resulted in a band of approximately 32 kDa under reducing conditions and 64 kDa, consistent with dimer formation, under nonreducing conditions. Soluble recombinant CD72-Flag protein was immobilized on a BIAcore sensor chip surface via an anti-Flag mAb. As shown in Figure 6B, when anti-CD72 mAb was injected over the chip, a typical profile of antibody binding was observed. We measured the binding affinity between CD100 and CD72 with the BIAcore system by injecting increasing concentrations of mCD100-Fc over the sensor surface containing immobilized CD72-Flag (Figure 6C). The mCD100-Fc samples were similarly injected over sensor surfaces containing only immobilized anti-Flag mAb (without soluble CD72-Flag protein) to measure the background response. For each mCD100-Fc concentration, the binding response at equilibrium was calculated by subtracting the response seen in the control flow cell from the response seen in the CD72-immobilized cells, resulting in a K_d value of $\sim 3 \times 10^{-7}$ M by equilibrium binding analysis. Neither CD5-Fc nor CD40-Fc could bind to recombinant CD72 protein immobilized on a BIAcore sensor chip (data not shown). Scatchard analysis using CD72-CHO cells and radioiodinated mCD100-Fc revealed that CD100 bound to CD72-CHO cells with a K_d of $\sim 3 \times 10^{-7}$ M (data not shown), which is comparable to the binding affinity of mCD100-Fc for WEHI-231 cells (Figure 3). Since CD72 belongs to the C-type lectin family, there is a possibility that CD72 may

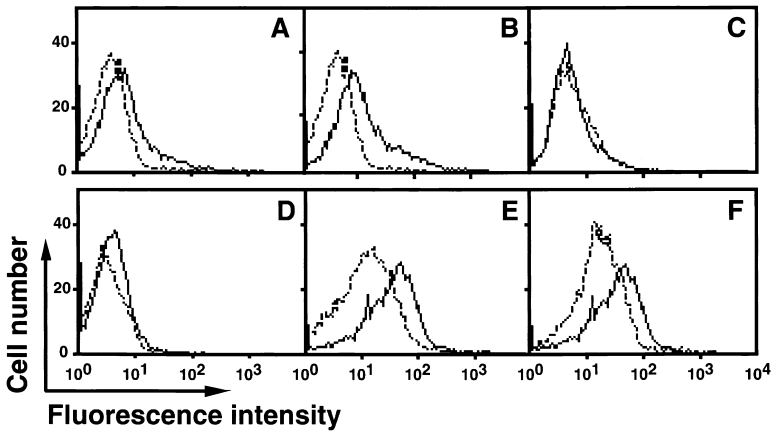

Figure 4. Flow Cytometric Analysis of CD100 Receptor

WEHI-231 cells (A), splenic B cells (B), thymocytes (C), splenic T cells (D), Con A (2 μg/ml) stimulated T cells (E), or T cell hybridoma 2B4 cells (F) were incubated with biotinylated mCD100-Fc (60 nM) in the presence of Fc block on ice for 1 hr, stained with FITC-conjugated streptavidin on ice for 20 min, and analyzed by flow cytometry (solid line). The profiles of cells stained with biotinylated mCD40-Fc (van Essen et al., 1995) (A and B) or human immunoglobulin Fc fractions (C–F) plus FITC-conjugated streptavidin are also shown (dotted line).

bind to carbohydrate moieties of CD100. While not totally excluding this possibility, treatment with glycosidases did not affect the binding of mCD100-Fc to B cells or CD72-CHO cells (Figure 6D).

CD72 has been shown to have ITIMs and to associate with SHP-1 via phosphorylated tyrosine residues of CD72 (Adachi et al., 1998; Wu et al., 1998). In addition, preligation of CD72 by anti-CD72 mAbs was shown to block tyrosine phosphorylation of CD72 in anti-μ stimulated B cells (Wu et al., 1998). We thus tested whether CD100 stimulation could reproduce the effects of anti-CD72 mAb on CD72-mediated signals. When WEHI-231 B cells were stimulated with anti-μ, CD72 was tyrosine phosphorylated and associated with SHP-1, as previously reported (Wu et al., 1998). Pretreatment of B cells with mCD100-Fc but not CD40-Fc (control fusion protein) could abrogate both tyrosine phosphorylation of CD72 and association of SHP-1 with CD72 (Figure 7A). Furthermore, when transiently expressed in COS7 cells, CD72 was constitutively tyrosine phosphorylated and associated with SHP-1. CD100 stimulation induced tyrosine dephosphorylation of CD72 and dissociation of SHP-1 from CD72 in these cells (Figure 7B). These results suggest that CD100 binding may turn off negative signaling through CD72 by dissociating SHP-1.

Discussion

CD72 Is a Functional Receptor for CD100 in the Immune System

We have demonstrated expression of two types of CD100 receptors in different cell types and have identified CD72 as a functional lymphocyte receptor for CD100. Neuropilins and plexins have been shown to be receptors for several members of the semaphorin family (He and Tessier Lavigne, 1997; Kolodkin et al., 1997; Comeau et al., 1998; Winberg et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999). Although neuropilins have very short cytoplasmic domains that contain no obvious signaling motifs, they are indispensable for repulsive semaphorin guidance (Nakamura et al., 1998), suggesting that they serve a critical role in assembling a receptor complex that includes a transmembrane signaling component. Plexins, which are more recently identified receptors for the semaphorin family, have been shown to form receptor complexes with neuropilins (Takahashi et al., 1999; Tamagnone et al., 1999). Human plexin-B1 has been recently cloned as a high-affinity binding receptor for CD100 (Tamagnone et al., 1999). We also detected high-affinity binding sites for CD100 on mouse renal tubular cells that express plexin-B1. *Plexin-B1* transcripts were detected in these mouse renal tubular cells but not in mouse primary lymphocytes. Our study therefore shows that CD72 is a novel type of semaphorin receptor distinct from neuropilins and plexins. Furthermore, B cell responses were severely impaired in CD100-deficient mice, but developmental defects were not apparent in other tissues (Shi et al., 2000 [this issue of *Immunity*]). It thus appears that CD100–CD72 interactions play a nonredundant role in the immune system and that if plexin-B1 has essential roles, it may have other ligands. However, we cannot completely exclude the possibility that, in addition to CD72, a neuropilin or plexin molecule other than plexin-B1 may be involved in mediating CD100 signals in the immune system.

CD72 has been a "wandering B cell antigen" until now. Although at one time the possibility was raised that CD72 might serve as a receptor for IL-4 (Yakura et al., 1986), this is now known not to be the case. CD72 has been suggested to be a ligand for CD5 because CD5 protein purified from detergent-solubilized T cells was shown to bind to CD72-expressing cells (Van de Velde et al., 1991). However, other reports have shown that recombinant CD5-Fc fusion protein consisting of the extracelluar domain of CD5 and human IgG bound not to CD72 but to a 35–37 kDa protein and/or two polypeptide chains of 77–80 and 37–40 kDa on activated B cells (Biancone et al., 1996; Bikah et al., 1998). Furthermore, we could not find specific binding between recombinant CD72 and CD5-Fc using the BIAcore instrument (data not shown). The present study has clearly shown that CD100 is a bona fide ligand for CD72. Some of the previously reported effects of anti-CD72 mAbs on B cells (Gordon, 1994; Tutt Landolfi and Parnes, 1997) are similar to those of CD100 transfectants and mCD100-Fc revealed by us and others (Hall et al., 1996). Furthermore, the binding affinity between CD100 and CD72 ($K_d = \sim 3 \times 10^{-7}$ M) is comparable to or higher than those of other adhesion or costimulatory molecules, such as CD2-CD58 ($K_d = \sim 1 \times 10^{-6}$ M) and CD80-CD28 ($K_d =$

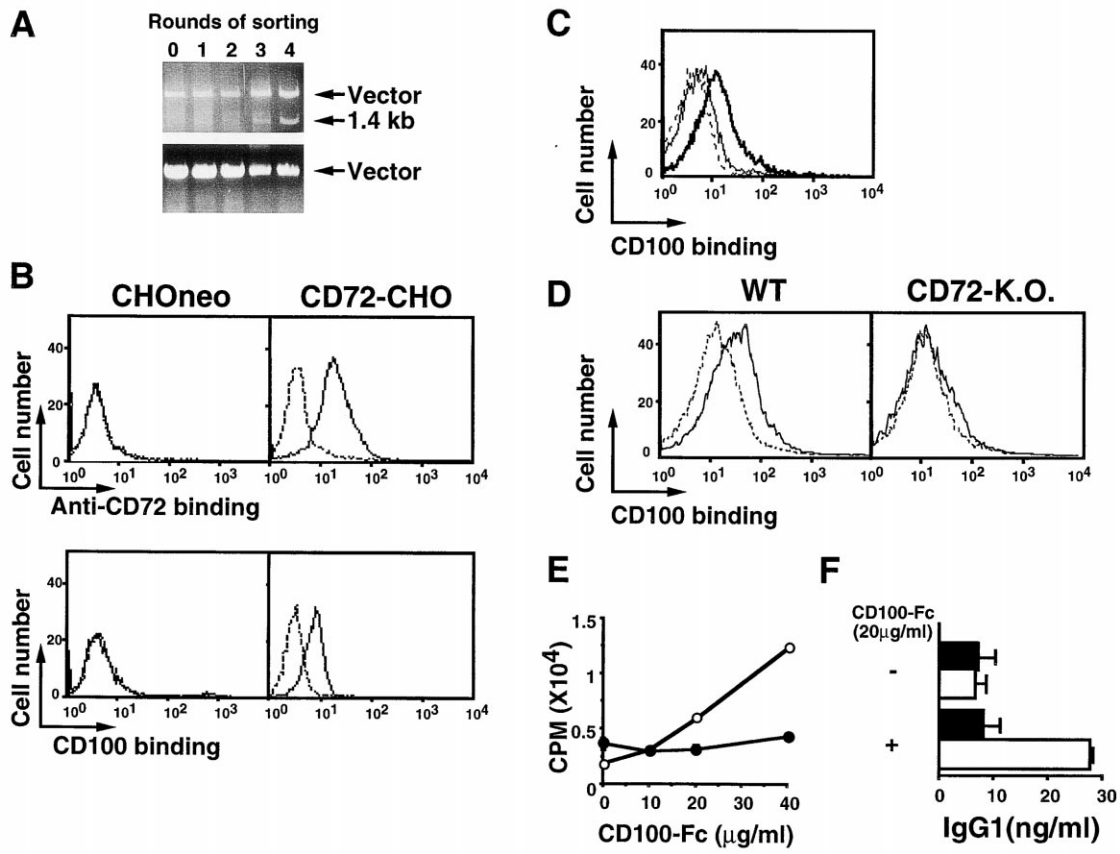

Figure 5. CD72 Is a Receptor for CD100

(A) Magnetic sorting enriched discrete 1.4 kb cDNA inserts (upper). No apparent bands were enriched after control sorting using biotinylated human immunoglobulin Fc fractions plus magnetic beads conjugated with streptavidin (lower). The band of 3 kb corresponds to the pME18S vector. Plasmid DNAs before 0 and after 1–4 magnetic sorting were digested with NotI and EcoRI to identify inserts and electrophoresed on 1% agarose gels.

(B) mCD100-Fc binds to CD72-CHO. (Upper) CHOneo or CD72-CHO cells were established as described in Experimental Procedures and stained with biotinylated anti-CD72 (IOT72-2) plus FITC-conjugated streptavidin (solid line) or FITC-conjugated streptavidin alone (dotted line). (Lower) mCD100-Fc binds to CD72-CHO cells. CHOneo or CD72-CHO cells were stained with biotinylated mCD100-Fc (solid line) or biotinylated mCD40-Fc plus FITC-conjugated streptavidin (dotted line).

(C) Anti-CD72 mAb specifically blocks the binding of mCD100-Fc to CD72 on B cells. B cells from DBA/2 mice were preincubated with 5 μg of anti-CD72 mAb (mouse anti-CD72; 9-6.1, Yakura et al., 1986) (thin line) or mouse IgG2b as an isotype control (thick line), stained with biotinylated mCD100-Fc plus FITC-conjugated streptavidin or FITC-conjugated streptavidin alone (dotted line), and analyzed by flow cytometry.

(D) mCD100-Fc does not bind to CD72-deficient B cells. Spleen cells were prepared from wild-type (left) or CD72-deficient mice (Pan et al., 1999) (right) and then stained with phycoerythrin-conjugated anti-B220 and biotinylated mCD100-Fc (solid line) or mCD40-Fc (dotted line) plus FITC-conjugated streptavidin. B220-positive cells were gated and analyzed for mCD100-Fc binding by flow cytometry.

(E) CD72-deficient B cells do not respond to CD100 stimulation. Wild-type (open circles or bars) or CD72-deficient (filled circles or bars) small resting B cells were prepared and stimulated with mCD100-Fc in the presence of anti-CD40 mAb (0.5 μg/ml) and IL-4 (10 U/ml) for 3 days for proliferation (E) or 7 days for immunoglobulin production (F).

$\sim 1 \times 10^{-6} \sim 10^{-7}$ M) or -CTLA-4 ($K_d = \sim 1 \times 10^{-6} \sim 10^{-7}$ M) (van der Merwe et al., 1997), all of which play significant roles in immune responses.

CD100 Turns off Negative Signals of CD72

We have shown here that CD100 stimulation induces tyrosine dephosphorylation of CD72, leading to SHP-1 dissociation from CD72 (Figure 7). These effects are consistent with previous studies showing that anti-CD72 mAb treatment blocks tyrosine phosphorylation of CD72 and recruitment of SHP-1 to CD72 in anti-μ stimulated B cells (Wu et al., 1998). CD72 has been postulated to play a role in B cell development and B cell responses as a negative regulator. CD72-deficient B cells are hyperproliferative, confirming this notion (Pan et al., 1999). Therefore, CD100 stimulation may enhance B cell responses by turning off the negative signaling effects of CD72. In fact, our accompanying report shows that the immunological phenotype of CD100-deficient mice is almost the mirror image of that of CD72-deficient mice (Shi et al., 2000). The homeostasis of the immune system is delicately controlled by both positive and negative regulators. Like CD72, many negative regulators, including killer inhibitory receptors, some Fc receptors, and CD22, contain ITIM motifs and are associated with various tyrosine phosphatases (Daeron et al., 1995; Tedder et al., 1997; Lanier, 1998; Falco et al., 1999). In most cases, ligand binding to these receptors transmits nega-

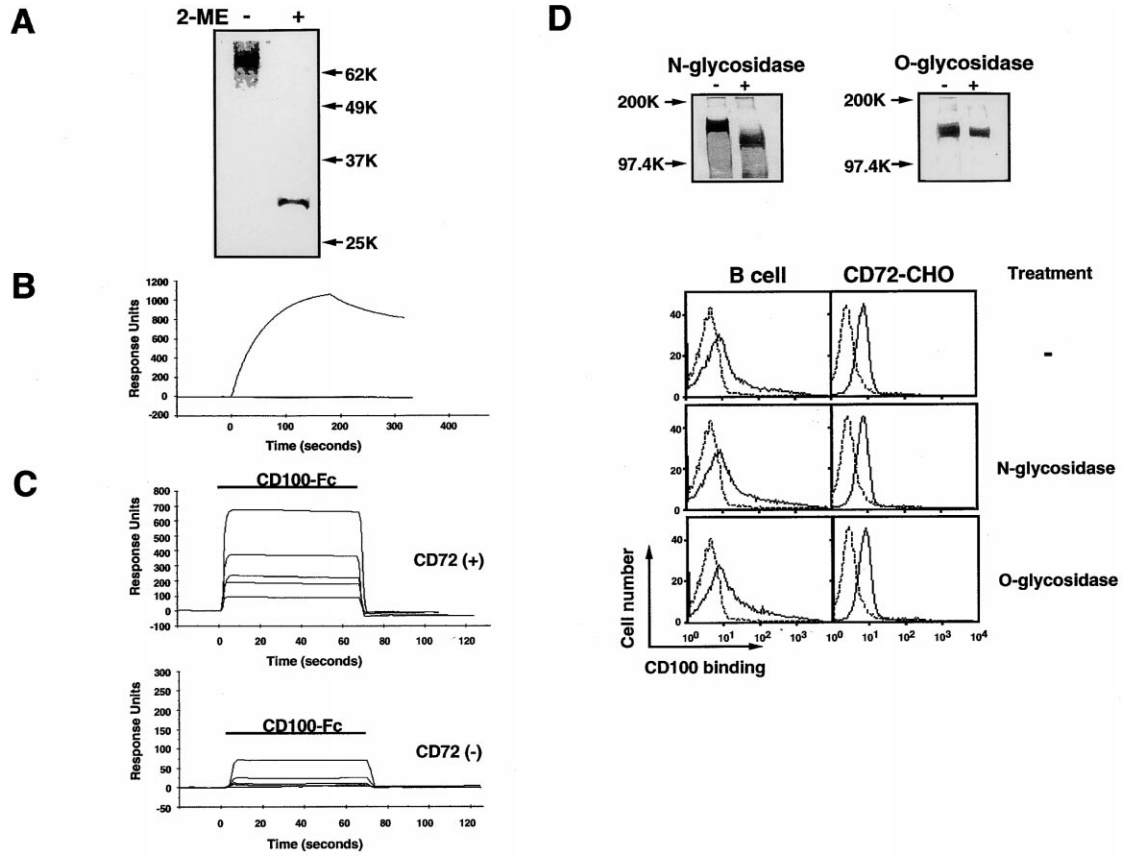

Figure 6. Properties of Binding between CD100 and CD72

(A) SDS-PAGE analysis of purified recombinant soluble CD72. Three hundred nanograms of purified recombinant soluble CD72 protein was separated by SDS-PAGE under reducing or nonreducing conditions and visualized by silver staining. Molecular weight markers (kDa) are shown on the right.

(B) Anti-CD72 mAb specifically recognizes recombinant soluble CD72-Flag protein. Anti-CD72 mAb (K10.6; mouse IgG2b, 10 μg/ml) was injected through the flow cell with recombinant CD72-Flag immobilized via anti-Flag mAb on a sensor chip. The recombinant CD72 protein was specifically recognized by anti-CD72 mAb. As a negative control, anti-CD40 mAb or mouse IgG2b (10 μg/ml) was injected using the same experimental conditions.

(C) Measuring the affinity of mCD100-Fc binding to recombinant CD72-Flag protein by surface plasmon resonance. A range of mCD100-Fc concentrations (54, 27, 18, 14, and 9 nM) was injected sequentially through the flow cell with recombinant CD72-Flag immobilized via anti-Flag mAb on the sensor chip (upper). As a control, the mCD100-Fc samples were injected through a flow cell with sensor chips coated with anti-Flag mAb alone without recombinant CD72-Flag (lower). The binding response at equilibrium was calculated by subtracting the response in the control from the response with the CD72-immobilized chip. The K_d was estimated by BIAcore evaluation software.

(D) Deglycosylation of mCD100-Fc does not block the binding of mCD100-Fc to CD72. After 24 hr treatment with N-glycosidase F or O-glycosidase, treated and nontreated mCD100-Fc were analyzed by SDS-PAGE. B cells or CD72-CHO cells were stained with glycosidase-treated or nontreated biotinylated mCD100-Fc proteins plus FITC-conjugated streptavidin (solid line). As a negative control, cells were stained with biotinylated human immunoglobulin Fc plus FITC-conjugated streptavidin (dotted line).

tive signals to suppress lymphocyte functions. Our findings may be the first example of ligand binding to a negative regulator leading to positive stimulation of lymphocytes. However, it is still unknown whether CD72 alone is responsible for CD100 signals or whether another unknown protein might associate with CD72 and also function as a signaling molecule. Thus, further studies to search for CD72-associated proteins other than SHP-1 may be necessary.

In conclusion, we demonstrate that the lymphocyte semaphorin CD100 binds to CD72, establishing the first endogenously expressed semaphorin receptor binding pair in the immune system. Moreover, the CD100–CD72 interaction results in strong B cell costimulation by the unique mechanism of turning off negative signaling. Our findings will provide not only novel insights into the regulation of humoral immune responses but also clues for the development of vaccines to potentiate antigen-specific antibody production.

Experimental Procedures

PCR-Based cDNA Subtraction

Aliquots of 1×10^8 WEHI-231 cells were stimulated with an anti-CD40 mAb, HM40-3 (PharMingen), for 8 hr. Total RNA from CD40-stimulated or unstimulated WEHI-231 cells was isolated using guanidinium isothiocyanate phenolic acid, and mRNA was purified by oligo(dT)-coupled magnetic beads (PolyA Tract mRNA Isolation Systems, Promega). cDNA synthesis and subtraction were performed using a PCR-Select cDNA subtraction kit (Clontech). The resulting cDNA fragments that were induced by CD40 stimulation were di-

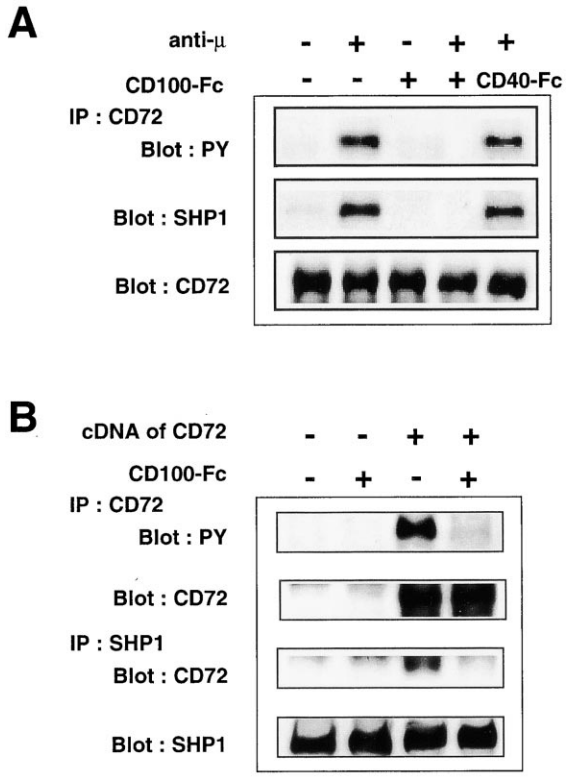

Figure 7. CD100 Induces Tyrosine Dephosphorylation of CD72 and Dissociation of SHP-1

(A) WEHI-231 cells (1×10^7 cells/lane) were pretreated with or without mCD100-Fc (60 μg/ml) or CD40-Fc (40 μg/ml) (control fusion protein) for 15 min on ice and then stimulated with $F(ab')_2$ anti-μ (10 μg/ml) for 1 min at 37°C.

(B) COS7 cells that were transfected with *CD72* cDNA by lipofection 2 days before were stimulated with mCD100-Fc for 1 min. Cell lysates (1% NP40) were immunoprecipitated with anti-CD72 (H-96) or anti-SHP-1 (C19) and blotted with anti-phosphotyrosine Abs (PY99), anti-SHP-1 (C19), or anti-CD72 (H-96) mAbs.

rectly ligated into a T/A vector (Novagen). The inserts were sequenced and analyzed by comparisons against GenBank and dbest databases.

Production of Soluble mCD100 Proteins

A truncated form of *mCD100* cDNA was prepared from a full-length *mCD100* cDNA derived from CD40-stimulated WEHI-231 cells by PCR using a pair of oligonucleotide primers containing a sense sequence including a SalI site (5′-GCTGTCGACTGTGTGCCCGTTG CTGAAGGCCT-3′) and an antisense sequence including a BamHI site (5′-GACGGATCCTACTTACTTTGCTTTGCTTGCTTGAGATA CACCGTCTTCTCTGA-3′) for mCD100-Fc, or an antisense sequence including a NotI site and Flag (DYKDDDDK) sequence (5′-AGGCGG CCGCTTACTTGAGATACACCGTCTTCTCTGA-3′) for mCD100-Flag. The resulting SalI–BamHI fragments or SalI–NotI fragments were used to replace the SalI–BamHI DNA fragment or SalI–NotI DNA fragments of the pEFBos human IgG1 Fc cassette (Suda and Nagata, 1994) to generate mCD100-Fc or mCD100-Flag proteins. To produce these soluble mCD100 proteins, stable P3U1 plasmacytoma transformants carrying the expression plasmid were established by electroporation. Briefly, aliquots of 10^7 cells were transfected with 50 μg of the plasmid DNA digested with HindIII for pEFBos-mCD100-Fc or mCD100-Flag or 5 μg of pMC1neo vector digested with BamHI by electroporation. After selection in RPMI medium containing 10% FCS and 0.3 mg/ml of G418 for 10 days, individual G418-resistant colonies were isolated and cloned. mCD100-Fc protein was purified from culture supernatants by protein A–Sepharose (Pharmacia), and mCD100-Flag protein was affinity purified by anti-Flag mAb (M2) conjugated with agarose (Sigma).

Transfectants

Stable *mCD100*- or *CD72*-expressing CHO cell transfectants were generated by introducing full-length Flag-tagged mCD100 or CD72 cDNAs in the pMC1neo vector using Lipofectamine Plus (Life Technologies). Transfectants expressing CD100 (CD100-CHO) or CD72 (CD72-CHO) were selected by anti-FLAG (M2, Sigma) or anti-CD72 (IOT72-2, Immunotech, Marseille, France) mAb and cloned. As a control transfectant, CHOneo was generated by transfection of CHO cells with the pMC1neo vector alone.

Antibodies

Anti-mCD100 mAb (BMA-12) was established as follows. Rats were immunized three times and boosted once with 100 μg of mCD100-Fc fusion protein. Rat splenocytes were fused with P3U1 cells, and 7 days later, hybridomas were tested for the production of specific antibodies by ELISA using mCD100-Fc and mCD100-Flag. Positive results were confirmed by flow cytometry using CD100-CHO. Anti-CD72 (H-96), anti-phosphotyrosine (PY99), and anti-SHP-1 (C19) antibodies were purchased from Santa Cruz Biotechnology, and $F(ab')_2$ fragments of goat anti-mouse IgM (μ chain) antibodies were from ICN Pharmaceuticals. Anti-CD72 mAb (K10.6) that recognizes the extracellular region of CD72 was purchased from PharMingen.

Flow Cytometric Analysis for Expression of CD100 and Its Receptor

Anti-mCD100 mAb (BMA-12; rat IgG1), mCD100-Fc, mCD40-Fc (kindly provided by Dr. D. Gray, Edinburgh, U.K.) (van Essen et al., 1995), or human immunoglobulin Fc fractions (Chemicon International, Inc.) were biotinylated using a biotinylation kit (Boehringer Mannheim). For flow cytometric analysis for CD100 receptors, aliquots of 10^6 cells were incubated with biotinylated mCD100-Fc (60 nM), mCD40-Fc (60 nM), or human immunoglobulin Fc on ice for 1 hr containing 5 μg/ml of Fc block (PharMingen). After washing with staining buffer, the cells were stained for 20 min with FITC-conjugated streptavidin (PharMingen). Cells were then washed and analyzed by a flow cytometer.

Assays for Proliferation and Immunoglobulin Production

Nonadherent splenic B cells from C57BL/6 mice (6–8 weeks) were isolated with a combination of anti-Thy1.2 (F7D5, Seroteck Ltd, U.K.) and rabbit complement (Wako, Japan). The remaining B cells were further fractionated through a Percoll gradient of 50%, 60%, 66%, and 70%, and cells at the interface between 66% and 70% were collected. The resulting small resting splenic B cells (1×10^5 cells/well) were stimulated with or without anti-CD40 mAb (1 μg/ml) and IL-4 (10 U/ml) in the presence of mCD100-Fc (20 μg/ml) or with paraformaldehyde fixed CHOneo or CD100-CHO cells (2×10^4 cells/well) in flat-bottomed 96-well microtiter plates for 3 days for proliferation or 7 days for immunoglobulin production. Cells were pulsed with 2 μCi [^{3}H]thymidine for the last 16 hr. Immunoglobulin production was estimated by ELISA (Kawabe et al., 1994).

Isolation of cDNA Fragments of Murine Plexin-B1

Based on the sequences conserved among human *plexin-B1*, *A1*, *A2*, and *A3* and mouse *plexin-A1*, *A2*, and *A3*, degenerate 5′(GTGAAA/GGTA/G/CCTA/GGAC/TTGTGAC/TAC) and 3′ (AACA GA/GTCA/GTCC/GACA/GAAT/CTTT/CTG) oligonucleotides were used for PCR amplification, using cDNA derived from the mouse renal tubular cell line, Prox24 (kindly provided by Drs. Y. Hirata and T. Sugaya, Tanabe, Japan) (94° for 1 min; 50° for 1 min; 72° for 1 min; 30 cycles). Amplification products were cloned into a T/A vector (Novagen) and sequenced. One 460 base pair amplification product encoded the mouse homolog of plexin-B1, exhibiting ~83% identical nucleotides and ~95% identical amino acid residues compared to human plexin-B1. Based on the sequence of mouse *plexin-B1*, RT–PCR, using 5′ (TGTGACACCATCTCCCAGGC) and 3′ (AGCCTT TGGCTTGGCTAACG) oligonucleotide primers, was performed (94°C for 30 s; 62°C for 30 s; 72°C for 30 s; 35 cycles).

Construction of the cDNA Library and Expression Cloning

2B4 cells were grown to 1×10^6 cells/ml in RPMI containing 10% FCS and then stimulated with Con A (2 μg/ml) for 18 hr. Total cellular RNA was isolated by guanidinium isothiocyanate gradient centrifugation, and mRNA was selected using oligo(dT)-coupled magnetic beads (PolyA Tract mRNA Isolation System, Promega). Double-stranded cDNA primed with oligo(dT) was synthesized using a SuperScriptII cDNA synthesis kit (Life Technologies). A BstXI adaptor (Invitrogen) was added to the cDNA, which was size fractionated by electrophoresis on a 1% agarose gel. cDNAs larger than 1.0 kb were recovered and ligated to BstXI-digested pME18S vector (Hara and Miyajima, 1992). *Escherichia coli* DH10B cells (Life Technologies) were transformed with the ligated DNA by electroporation. Aliquots of 2.0×10^7 independent clones were used to transfect COS7 cells. COS7 cells (40×10 cm dishes) were transfected with plasmid DNAs using lipofectamine plus (Life Technologies). Three days after transfection, the cells were harvested, resuspended to a concentration of 5×10^6 cells/ml in PBS containing 5% FCS, 2.5 μg/ml of Fc block (PharMingen), and 5 μg/ml of biotinylated mCD100-Fc or biotinylated human immunoglobulin Fc, and incubated on ice for 1 hr. The cells were washed with ice-cold PBS and suspended to 5×10^6 cells/ml in PBS containing Dynabeads M-280 streptavidin (Dynal A.S). After incubation for 30 min, the cells were washed with ice-cold PBS ten times using a Magnetic Particle Concentrator (Dynal A.S). The extrachromosomal plasmid DNA was extracted from binding cells by the Hirt method (Seed and Aruffo, 1987). The plasmid DNA was introduced into *E. coli* DH10B cells (Life Technologies) by electroporation and then applied to the second (18×6 cm dishes), third (12×6 cm dishes), and fourth (6×6 cm dishes) transfection by protoplast fusion (Seed and Aruffo, 1987). Magnetic sorting was repeated four times as described above.

Surface Plasmon Resonance

Binding experiments were performed by surface plasmon resonance on a BIAcore instrument. The affinity was measured by equilibrium binding analysis rather than by binding kinetics because of the relatively low affinity interaction between CD100 and CD72. All experiments were performed using HBS buffer (25 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20) supplied by BIAcore. Anti-FLAG M2 antibody was covalently coupled by primary amine groups to the carboxylmethylated dextran matrix on a research grade CM5 sensor chip (BIAcore) using the Amine Coupling Kit (BIAcore), following which recombinant CD72-Flag protein was injected and trapped on the anti-FLAG-coated sensor chip. The K_d value was estimated by BIAcore evaluation software.

Generation of Recombinant Soluble CD72 Protein

The signal peptide of CD59 was used for secretion of soluble CD72 protein. The cDNA of *CD72* encoding the full length of the extracellular region with sequence encoding the Flag peptide fused to the sequence representing the amino terminus was inserted into a CD59 signal sequence cassette (kindly provided by Dr. K. Ohishi, Osaka University, Japan). The construct was introduced into COS7 cells by Lipofectamine Plus, and soluble CD72-Flag protein was purified from the culture supernatants using anti-Flag mAb (M2)-conjugated agarose.

Glycosidase Treatment

To determine the potential role of glycosylation of CD100 in CD100–CD72 interactions, 25 μg of mCD100-Fc was subjected to N-glycosidase F (Takara Shuzo Co., Japan) or O-glycosidase (Boehringer Mannheim) hydrolysis, according to each vendor's recommendations, in nondenaturing conditions. Untreated and glycosidase-treated mCD100-Fc were compared for mobility by SDS-PAGE and for CD72 binding activity by flow cytometry.

Immunoprecipitation and Western Blotting

Cells were lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM Na_3VO_4, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and protease inhibitors. For immunoprecipitation, the cell lysates were precleared with protein G–Sepharose beads (Amersham Pharmacia Biotech), followed by incubation with protein G–Sepharose beads plus anti-CD72 (H-96) or anti-SHP-1 (C19) for 3 hr at 4°C. After washing with the lysis buffer four times, immunoprecipitates were subjected to SDS-PAGE and then electrophoretic transfer to nitrocellulose membranes. Membranes were immunoblotted with anti-CD72 (H-96), anti-SHP-1 (C19), or anti-phosphotyrosine Abs (PY99), and the blots were developed by enhanced chemiluminescence (ECL) reagent following the manufacturer's protocol (Amersham Pharmacia Biotech).

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